AD_____

GRANT NUMBER DAMD17-96-1-6315

TITLE: Fluid Transport Mechanisms in Breast Gross Cystic Disease

PRINCIPAL INVESTIGATOR: Sheldon Miller, Ph.D.

CONTRACTING ORGANIZATION: University of California
Berkeley, CA 94720-5940

REPORT DATE: September 1997

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

19980310 061

DTIC QUALITY INSPECTED 2

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 1997	Annual (1 Sep 96 - 31 Aug 97)

4. TITLE AND SUBTITLE
Fluid Transport Mechanisms in Breast Gross Cystic Disease

5. FUNDING NUMBERS
DAMD17-96-1-6315

6. AUTHOR(S)
Sheldon Miller, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of California
Berkeley, California 94720-5940

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

The goal of this research is to develop a pharmacological strategy for reducing or eliminating the pathological accumulation of fluid that occurs in breast gross cystic disease (GCD). We have begun an analysis of the transport proteins and intracellular signaling mechanisms that mediate the movement of fluid (and ions) across confluent monolayers of human cultured mammary epithelial cells beginning with a well-characterized breast cancer mammary cell line (**31EG4**). The electrophysiology data obtained using 31EG4 cultures provides evidence in human mammary cells for apical membrane Cl channels (cAMP and Ca^{2+}-activated) and amiloride sensitive Na channels. **The present preliminary experiments have provided the first measurements of fluid transport across any mammary epithelia.** In 11 cultures the mean rate of fluid transport (J_V) was 4.6 ± 8.9 $\mu l \cdot cm^{-2} \cdot hr^{-1}$ and the mean transepithelial potential (TEP) and resistance (R_T) were -6.4 ± 4.1 mV and 777.7 ± 444.7 $\Omega \cdot cm^2$, respectively. The variation in J_V is large because these tissues are capable of absorbing (6/11) or secreting fluid (5/11). The present data show that these transformed cultures have an excessive capability for fluid secretion via cAMP- activated Cl channels located in their luminal or apical membranes. We also showed that the amiloride sensitive Na absorption pathway could also contribute to excessive secretion if it were down regulated or blocked (Fig 12). How can this excessive capability for fluid secretion be mitigated or avoided? Clearly we must use pharmacological or gene therapy techniques to decrease secretion via the cAMP - dependent pathway or up-regulate or increase fluid absorption via the apical membrane Na - dependent pathways.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer	23
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

____ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

____ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

SM ____ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

____ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

____ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

____ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Sheldon Miller 9/28/97

PI - Signature Date

Table of Contents

INTRODUCTION

The goal of this research is to develop a pharmacological strategy for reducing or eliminating the pathological accumulation of fluid that occurs in breast gross cystic disease (GCD). Over the last year, we have begun an analysis of the transport proteins and intracellular signaling mechanisms that mediate the movement of fluid (and ions) across confluent monolayers of human cultured mammary epithelial cells beginning with a well-characterized breast cancer cell line. We hope eventually to be able to analyze cells cultured from: (1) reduction mammoplasties in normal individuals; and (2) patients with gross cystic disease. In all cases, we will use antibodies and immunocytochemistry to identify the transport proteins that are located on the apical and basolateral membranes of these cells. At the same time, in a complementary set of experiments, confluent monolayers of each cell type will be mounted in modified Ussing chambers to measure the transport rates of the pumps, cotransporters, exchangers, and channels that are located at each membrane. **The present preliminary experiments have provided the first measurements of fluid transport across any mammary epithelia.** In particular, the magnitude and direction of the fluid transported across each preparation has been directly measured. As shown in the preliminary data (see below) we have been able to make solution composition changes and add secretagogues or transport inhibitors to the apical or basal baths in order to help identify which particular apical or basolateral membrane proteins and/or which signaling pathways mediate the vectorial transport of fluid across **31EG4** mammary cell line (Sjaasted *et al.*, 1993**).**

BODY

Background/Significance

GCD is the most common benign breast disease and its etiology is unknown. Seven to ten percent of women in Western countries develop macrocysts of the breast, mainly in the premenopausal decade, and there is now strong evidence to indicate that these women are at a higher risk of later developing breast cancer, at a rate 2 - 4 times that of the general population (Angeli et al., 1994; Leis, 1993; Bodian, 1993; Bodian et al., 1992; Bundred et al., 1991; Ciatto et al., 1990).

In breast gross cystic disease (GCD) there are two types of epithelial cells that line the cysts, apocrine (tall, columnar) and attenuated (flat); it is the apocrine cells that serve as a marker for precancerous changes in the surrounding tissue (Leis, 1993; Petrakis et al., 1993). These cysts can be formed in one of two ways: either by a fibrocystic obstruction in the duct or by excessive fluid secretion across the ductal-alveolar epithelia (Dogliiotti et al., 1990). In the former case cysts do not usually reoccur after aspiration, but in the latter case - cysts frequently do reoccur (Angeli et al., 1987; Molina et al., 1990). Recent evidence suggests that the apocrine cells may provide the paracrine or autocrine signals that lead to the development of breast epithelial hyperplasia (Athanassiadou et al., 1992; Leis, 1993; Cassoni et al., 1995). If as in many organ systems, the progression from benign to malignant epithelial -cell phenotype were in part regulated by inhibitory signals then fluid accumulation in the

cysts would **reduce the activity** of these inhibitory signals and speed the onset of tumor growth. For example, in breast carcinomas, E-cadherin mediated adhesion between cells is significantly reduced because the expression level of this protein is reduced (Shiozaki et al., 1991; Eidelman et al, 1989).

Breast cyst fluid from GCD patients contains many different chemical components at activity levels that are quite unusual for any ventricular space. These abnormal secretory products include electrolytes, hormones, growth factors, cytokines, immunoglobulins and other proteins (Bradlow et al., 1985; Miller, 1989; Wick et al, 1989; Molina et al., 1990; Dogliotti et al., 1990; Reed et al., 1992; Lai et al., 1993; Angeli et al, 1987, 1992, 1994; Cassoni et al., 1995). Approximately 70% of the total protein content of the breast GCD fluid is made up of only three proteins with molecular weights of 15, 24 and 44kd (gross cystic disease fluid proteins - GCDFP- 15,24,& 44). Of these three polypeptides, GCDFP-15 is the one consistently present in carcinomas of the breast (Wick et al., 1989; Mazoujian et al., 1989; Petrakis et al., 1993; Cassoni et al., 1995). We will begin by measuring the activity GCDFP-15 and its ability to alter fluid transport rate because: (1) its secretion is increased in breast cancer (Umaar et al., 1994); (2) it is mitogenic for normal human and breast cancer cell lines (Cassoni et al., 1995), but **not** colon carcinoma cell lines (HT29 or NIC-H716), neuroblastoma cell line-IMB32 or NIC-H209 (small cell lung carcinoma cells). The chemical activity of this secreted protein and its role in the development of breast epithelia hyperplasia may be regulated, in part, by the amount of fluid secreted into the cyst.

The electrolyte composition of gross breast cysts are quite abnormal compared to most secretions (high K and low Na, Cl & pH; Angeli et al., 1994; Molina et al., 1990; Dixon et al., 1984) but it bears a striking resemblance to the endolymph fluid produced by the apocrine - like dark cells that line the semicircular canal of the ear (Marcus et al., 1992). Because the dark cells also secrete some of the same proteins (GCDFP-15), they provide a possible model for fluid (and ion) transport across mammary cells. Previous work using mammary cells from animal tissues also provides a helpful starting point (Shennan, 1990) but most of these studies were carried out using isolated epithelial cells, from mouse or bovine mammary glands or vesicle preparations (Shennan et al., 1994; Furuya et al., 1993; Shennan, 1992). In one case, confluent monolayers were used to study the influence of hormonal regulation on the polarized distribution of Na/H exchange and $Na{\cdot}HCO_3$ cotransport (Sjaastad et al., 1993). Some ion transport information has been obtained using confluent monolayers of cultured mouse mammary epithelia (Bisbee, 1981a,b), but nothing is known about the mechanisms that drive fluid secretion across the epithelia that line the ducts and lobules of the mammary glands.

In most gland systems fluid secretion is driven by a chloride transport pathway consisting of an apical membrane Cl channel and a basolateral membrane Na, K, 2Cl cotransporter. There is some evidence that this is also true for two of the human breast cancer cell lines, MCF-7 and BC19/3 (Altenberg et al., 1994). These authors showed that intracellular Cl concentration in both cell lines is quite high, $\approx$ 40 mM, strongly suggesting that chloride is actively accumulated, perhaps via furosemide-sensitive

Na,K,2Cl cotransporters (Shennan, 1989, 1990); Cl efflux in MCF - 7 cells occurs via Ca^{2+} -dependent Cl channels (Flezar and Heisler, 1993). These results suggest the presence of a chloride transport pathway that could drive fluid secretion into the ducts. Therefore our first experiments have probed for the presence of apical membrane Cl channels and basolateral Na, K,2Cl cotransporters, because this pathway will most likely provide the basis for a therapeutic approach to the prevention of fluid-filled cysts in gross cystic disease patients.

Previously we determined the membrane and intracellular signaling mechanisms that regulate fluid transport across the retinal pigment epithelium (Hughes et al., 1984; Edelman and Miller, 1991; Joseph and Miller, 1992; Edelman et al., 1994; Gallemore et al., 1997). We also demonstrated that these same mechanisms serve to control the hydration of the small extracellular space that lies between the RPE apical membrane and the distal portion of the neural retina (Bialek and Miller, 1994; Peterson et al., 1997). Recently we provided the first measurements of fluid transport across monolayers of cultured human airway surface and gland cells and determined some of the mechanisms that could provide therapeutic hydration in the airways of cystic fibrosis patients (Jiang et al., 1993; Jiang et al., 1997). Based on this experience, I believe that we will be able to develop a pharmacological strategy that can be used to prevent or reduce the fluid that accumulates in the mammary gland ducts during gross cystic disease. The potential benefits of preventing this accumulation of fluid would be three-fold: (1) a possible reduction in cancer risk or disease progression; (2) a reduction or removal of the pain that inevitably accompanies the fluid - induced intracystic tension; (3) the removal of an impediment that continues to cause misdiagnosis of breast cancer by primary care physicians (Leis, 1993).

Methods

Human Tissue Samples: Normal human mammary tissue was obtained from the Cooperative Human Tissue Network (CHTN). The mammary epithelial cells were isolated using the following collagenase digestion and percoll gradient procedure (Richards, et al., 1984; Imagwa et al., 1994). Tissue was minced for 10 minutes then placed in 50 mls of Hepes-buffered 199 media (plus antibiotics) with 0.1% collagenase. The sample was dissociated for 1.5 hrs at 37°C (with shaking). The samples were checked periodically for clumps. Once dissociated, the sample was spun for 5 minutes at 1000 rpm in a sterile 50 ml tube. The suspension of tissue and lipids on top of the media was removed and saved. The rest of the media was discarded while the pellet of cells on the bottom of tube was saved. To the saved suspension 40 mls of Hepes-buffered 199 media and 200ul of DNase was added. This sample was then vortexed for 2 minutes and spun again for 5 minutes at 1000 rpm. After the spin the media was removed and the cells pooled with the other pellet. The sample was resuspended, vortexed for 5 minutes and then spun again for 5 minutes at 1000 rpm. The cells were then spun in a percoll gradient. The epithelial cells were removed and washed in MEM media. The mammary epithelial cells were seeded on transwells at a density of 10^6 cells/cm^2 and allowed to grow into a confluent monolayer on a floating collagen gel.

Prolactin, cortisone and insulin (5µg/ml), was used to induce to lactation in some samples.

Mouse tissue samples: Mammary tissue was removed from virgin balb c mice. Isolation of the mammary epithelial cells was then initiated by mincing the tissue. The minced tissue was placed in 50 mls of Hepes-buffered 199 media (plus antibiotics) with 0.1% collagenase and dissociated for 1.5 hrs at 37°C (with shaking). The samples were checked periodically for clumps. Once the tissue was dissociated the sample was spun for 5 minutes at 1000 rpm in a sterile 50 ml tube. On the top of the media was a tissue/fat suspension. This suspension was removed and saved. The rest of the media was discarded while the pellet on the bottom of tube was saved. To the saved suspension 40 mls of Hepes-buffered 199 media and 200ul of DNase was added. This sample was then vortexed for 2 minutes and spun again for 5 minutes at 1000 rpm in a sterile 50 ml tube. After the spin the media was removed and pooled with the pellet. This pellet was then resuspended, vortexed for 5 minutes and then spun again for 5 minutes at 1000 rpm. The cells were then run through a percoll gradient. The epithelial cells were removed, washed in MEM media. The cells were seeded on transwells at a density of 10^6 cells/cm^2 and allowed to grow into a confluent monolayer on a floating collagen gel.

Human culture samples: MCF-7 cells were obtained from the American Type Culture Collection(ATCC) and Professor Gary Firestone. (UC Berkeley) They were grown in Minimal essential media (MEM) with Earle's Salts and non-essential amino acids (Gibco), glutamine, 10% Fetal bovine serum (FBS), 10 ug/ml bovine insulin, streptomycin and penicillin. These cells were seeded at $1x10^6$ cells/flask. Upon reaching confluencey, cells were plated onto Transwells at a density of 2.5 x 10^5 cells/well. Transwells were coated with laminin (l.5 µg/well), collagen (20 µg/well), human extracellular matrix (20 µg/well), or nothing. Plates were seeded at $6x10^2$ cells/well, $6x10^3$ cells/well, and $6x10^4$ cells/well. Six of these wells were maintained with the normal media, plus epidermal growth factor (EGF, 0.5 µg/mL). 31EG4 mouse mammary cells obtained from Professor Firestone were maintained in DMEM/F12, 5% FBS, 5µg/mL insulin, 50 µg/mL gentamicin sulfate. These cells were seeded into a T-25 flask at $6x10^6$ cells. Transwells were seeded at a density of $5xl0^5$ cells/well. 31EG4 cells were treated with dexamethasone (1 µM) to induce tight junction formation. Transepithelial resistance measurements were taken with an EVOM (Epithelial Voltammeter, World Precision Instruments, New Haven CT).

Electrophysiology: Transwells with confluent monolayers and an R_T over 100 $\Omega \cdot cm^2$, were then used for the electrophysiology measurements. The tissue was mounted on a nylon mesh support and clamped into a modified Ussing chambers, as previously described. The transepithelial potential (TEP) was measured by calomel electrodes in series with Ringer-agar bridges. TEP is the voltage difference between the apical and basal bath electrodes (Vb-Va). The transepithelial resistance (R_T) were obtained by passing current pulses, usually of 2µA across the tissue and measuring the change in TEP ($R_T = \Delta TEP/\Delta I$). The $\Delta TEP/\Delta I$ was multiplied by the area of the chamber (0.075 cm^2) to obtain the units $\Omega \cdot cm^2$.

The control ringer (1 liter) was made of: 5g KCl, 0.8g $MgSO_4 \cdot 7H_2O$, 113.4g NaCl, 26.2g $NaHCO_3$, 1.0g NaH_2PO_4 anhydrous, 5.6g D-glucose, 5g taurine, and 1.8g $CaCl_2$, at pH 7.4. For 8.6 mM Cl ringer, 113.4 g Methanesolufonic Acid was substituted for NaCl. MCF-7 cells were treated with low Cl ringer, a cAMP cocktail, made up of 500 µM IBMX, 100 µM CPT, and 12µM forskolin, as well as 10-8 M epinephrine, and 2 mM barium chloride. 31EG4 cells were treated with the cAMP cocktail, 10µM amiloride, and 20 µM ionomycin.

Fluid and ion transport: Transepithelial fluid flows were measured using an extremely sensitive capacitance probe technique developed in this laboratory (Hughes et al, 1984; Jiang et al., 1993) in which a sheet of epithelium (0.5 cm^2 exposed area) is mounted between two water-impermeable Kel F half-chambers. A very sensitive oscillator circuit is connected to a pair of capacitance probes placed on each side of the tissue to measure the changes in capacitance between the probe tips and the fluid meniscuses that are produced as ion-linked fluid is osmotically driven across the tissue from its apical to basolateral surfaces (or vice versa). Fluid movement across the epithelium is recorded by the changes in probe output measured as a voltage difference. Ports in the bottom of the half-chambers allow for solution composition changes. Voltage sensing and current-passing bridges built into each half chamber permit continuous monitoring of transepithelial voltage (V_T) and resistance (R_T), the latter from the voltage deflections in response to current pulses of known magnitude. This technique has an accuracy of ± I nl/min, corresponding to a fluid rate less than 1/10th of the average baseline fluid transport rate seen in these experiments.

PCR: RT-PCR was used to see if phospholamban was present in human mammary tissue. . Human mammary cells were isolated from a fresh tissue received from CHTN. The cells were was homogenized and total RNA was extracted using the RNAzol B method. cDNA was made using 0.5 mg of total RNA, oligo dt primers, and MMLV reverse transcriptase. Thirty-seven PCR cycles were run on the CDNA using primers designed to cover the entire phospholamban coding region and produce a 463 base pair band. The PCR product was run on a 1.5% agarose gel.

Results

Cell Culture: Initially primary human and mouse mammary epithelia cultures grew well in the transwells. As the epithelial cells grew into confluent monolayers they exhibited a transepithelial resistance 400-600 $\Omega \cdot cm^2$ and a TEP of 8-10 mV (apical side negative). But as the cells formed monolayers in the collagen, the cultures had a tendency to round up. This made it impossible to mount them as flat sheets in an Ussing chamber. Removing the monolayer from the rigid support of the transwells also caused the tissue to curl. The thickness of the collagen matrix that the cells grew in also made it difficult to mount in the chamber. This inability to mount a functional primary tissue monolayer lead us to examine the mammary cells lines MCF-7 and 31EG4.

Electrophysiology Experiments: The use of MCF-7 and 31EG4 allowed us to test the transport model that we have developed for mammary cells (see Figure 1). We have used our electrophysiology and fluid transport measurements to test the validity of this model. First, the seeding conditions for both cell lines had to be determined. When the MCF-7 cells were subcultured onto uncoated Transwells, the cells often aggregated together; some even formed gland like structures. Transwells were then coated with a variety of different substrates- laminin, collagen, and human extracellular matrix- to see if the cells would adhere better. Cells were more likely to form confluent monolayers on Transwells with no added substrate. Transwells were also seeded at a variety of densities, $6x10^2$ cells/well, $6x10^3$ cells/well, and $6x10^4$ cells/well. The density of the seeding did not significantly effect the ability of the cells to grow in a flat monolayer. Since, epithelial growth factor (EGF) has been shown to help the proliferation of MCF-7 cells (Resnicoff and Medrano, 1989), some of the wells were maintained in media plus EGF. These cells did not show any significant increase in the rate of proliferation over those grown without EGF. When the MCF7 cells were seeded on Transwells at $2.5x10^4$ cells/well they had an average R_T of 70 -135 $\Omega \cdot cm^2$, which were variable and usually not high enough to carry out electrical or fluid transport measurements. This result suggested that the junctional complexes of the monolayer were not well formed. Our preliminary data using this preparation is described below in Figures 2-6.

We were more successful, however, in growing functional monolayers of 31EG4 cells. These cells were seeded on Transwells at $5x10^5$ cells/well and had an average R_T of 100 $\Omega \cdot cm^2$. In contrast, those treated with dexamethasone had an average R_T of 1000 $\Omega \cdot cm^2$. Wells that looked 100% confluent and had an $R_T \geq 100$ $\Omega \cdot cm^2$ **could be** used in the electrophysiology and fluid transport apparatuses to measure changes in voltage, resistance and fluid. Our previous work in the retinal pigment epithelium (eye epithelium) and in the airways are consistent with this finding.

Figures 2-6 all show that MCF-7 have an apical positive TEP indicating that the apical membrane is more hyperpolarized compared to the basolateral membrane. In most epithelia that suggests the predominance of K channels at the apical membrane as indicated in the model slide shown in Figure 1.

Figure 2 shows that perfusing the **basolateral** side of MCF-7 cells with 8.6 mM Cl ringer (low Cl) caused an increase in TEP (V_B-V_A). There was also an increase in R_T . Removal of the low Cl ringers resulted in recovery of both the TEP and R_T. Figure 3 shows that when the apical side was bathed in a low Cl Ringer the opposite effect seen; TEP decreased and the R_T and these responses are reversible. The low Cl -induced changes in TEP and resistance are consistent with the presence of Cl channels at the apical and basolateral membranes, however this conclusion is not definitive because there could have been contributions from potentials generated in the shunt pathway. The addition of cAMP cocktail to the apical side of MCF-7 cells elicited no response (Fig 4). If there had been a response it would suggest the presence of CFTR, a cAMP-dependent Cl channel, suggesting that the low Cl -induced diffusion potentials may have been generated by other types of Cl channels, perhaps Ca^{2+}-activated as in many other epithelia. This hypothesis was partially tested by the addition of epinephrine to the apical or basal bathing solutions. This catecholamine activates β or α_1 adrenergic receptors in

bovine and human retinal pigment epithelium (or RPE; Joseph and Miller, 1992), and is metobotropically coupled to an elevation in cell Ca^{2+} and to an increase in plasma membrane Cl conductance. Figures 5 and 6, however, show that epinephrine had little effect at either the apical or basolateral membranes of MCF-7 cells.

We next turned to the 31EG4 cells because they could maintain a much better electrical seal, by a factor of 3 or 4 compared to the MCF-7 cultures. This improved electrical seal indicates that the polarity of these cells would be much more intact than the MCF -7's and that any changes in voltage and resistance could be more easily observed. Another potential defect of low resistance monolayers is that the chemical composition changes that are made on one side of the epithelium **cannot** be isolated from the opposite side. It is important to maintain this isolation if one wants to separately analyze the transport mechanisms on the apical and basolateral sides of the tissue.

Figures 7-10 show that 31EG4 cultures can have a negative TEP. Figure 7 shows that in striking contrast to the MCF-7's, addition of cAMP cocktail to the apical bathing solution 31EG4 cells caused a rapid increase in TEP toward more negative potentials. This increase was followed by a slower decrease toward zero; there was little change in total tissue resistance. This result is consistent with the presence of a cAMP-dependent Cl channel at the apical membrane, perhaps CFTR. The absence of a change in R_T suggests that more than one channel resistance was altered as well as the resistance of the paracellular pathway. A smaller decrease in TEP was seen when the cAMP cocktail was added to the basal bath (Fig 8, a result also consistent with the presence of CFTR at the apical membrane. In order to make that conclusion definitive we will have to repeat these experiments with microelectrodes in the cells. Those experiments will be carried out in the next several months.

Figure 9 shows another potentially important result. Apical amiloride decreased the TEP and increased R_T consistent with the presence of amiloride sensitive sodium channels at the apical membrane. Furthermore, Figure 10 shows that ionomycin, a calcium ionophore which elevates cell Ca^{2+} significantly increased TEP and decreased R_T exactly as expected if the apical membrane contained a Ca^{2+}-activated Cl channels.

***Fluid Transport Experiments*:** In the next series of experiments we mounted the 31EG4 monolayers in a specially designed fluid transport chamber (Hughes et al., 1984; Jiang et al., 1993) that allowed us to simultaneously measure TEP, R_T AND fluid transport across the epithelium. These tissues differed form the ones above in that they were cultured in dexamethasone, prolactin and insulin - factors that have been previously shown to improve cell polarity, especially with respect to the up regulation of tight junction proteins and basolateral membrane Na/H exchanger (Sjaasted MD, et al., 1993**).** The preliminary data suggests that these cells have a consistently higher resistance and voltage (see Table1) than the ones cultured only in DMEM/F12 and dexamethasone. **It should be emphasized that these are the only extant measurements of fluid transport across any mammary cell preparation.**

Figure 11 summarizes an approximately 3 hr experiments in which we carried out voltage, resistance and fluid transport changes following the addition and removal

of cAMP to the apical bath. The first thing that one should note is the size of the TEP and resistance in control Ringer, 6-7 mV and 800-900 $\Omega \cdot cm^2$, respectively. These values indicate a significant transport potential and fluid transport rate. The fluid transport rate in control Ringer was $\approx$ 2 $\mu l \cdot cm^2 \cdot hr^{-1}$ and then slowly decayed toward zero. Elevating cell cAMP caused a massive increase in steady-state fluid secretion to $\approx$ - 7 $\mu l \cdot cm^2 \cdot hr^{-1}$ and that increase along with the increase in TEP and the decrease in R_T almost certainly means that the apical membranes of these cells contain a large amounts of cAMP-dependent Cl channels.

The results summarized in Figure 12 are also very striking. In control Ringer the TEP and R_T are again very large, -10 mV and 1500 $\Omega \cdot cm^2$. In this experiment the fluid transport rate in control Ringer was initially -1 $\mu l \cdot cm^2 \cdot hr^{-1}$ and then decayed toward zero (note the following control held steady at -1 $\mu l \cdot cm^2 \cdot hr^{-1}$). The addition of amiloride to the apical bath caused a rapid and large decrease of TEP (-10 to -3 mV) and a large increase in R_T ($\approx$ 300 $\Omega \cdot cm^2$). At the same time J_V increased to $\approx$ -2 $\mu l \cdot cm^2 \cdot hr^{-1}$. What these results help demonstrate is that fluid transport across the mammary cell epithelial layer is determined by a balance of two processes, one absorptive and the secretory. Blocking the amiloride - sensitive Na channels helped decrease absorption and therefore net secretion increased! The effects of amiloride at the basolateral side produce a different set of results perhaps because at that membrane it is inhibiting the Na/H exchanger known to be located there (Sjaasted *et al.*, 1993). The latter conclusion remains to be much more rigorously tested.

Conclusions

Electrophysiology Experiments: The electrophysiology data obtained using 31EG4 cultures is important because it provides the first evidence in human mammary cells for apical membrane Cl channels (cAMP and Ca^{2+}-activated) and amiloride sensitive Na channels. The existence of these transport mechanisms provide the physiological basis for understanding how fluid and milk are transported into the mammary ducts. The excessive secretion of fluid seen in breast cystic disease could be pharmacologically or genetically reduced by upregulating fluid absorption out of the ducts into the blood via the amiloride sensitive Na channels or down regulating secretion via the apical membrane Cl channels.

***PCR*:** Phospholamban has been shown to be an important protein for modulating the levels of intracellular calcium cardiac and skeletal muscle contraction rates. Recently, we have used RT-PCR and immunocytochemistry to find the message for this protein and localize it in the RPE and the airways epithelium. In the retinal pigment epithelium this protein helps regulate the rate of fluid absorption out of the eye. Thus far we have found no evidence for this message in MCF-7 cells. We are in the process of examining this question for 31EG4's.

Fluid Transport Experiments: The preliminary fluid transport data that we have generated provides important results for understanding mammary cell pathophysiology and suggest several mechanisms that could help contribute to the observed abnormalities in fluid transport that occur in breast cystic disease. The present data show that these transformed cultures have an excessive capability for fluid secretion via cAMP- activated Cl channels located in their luminal or apical membranes. It also seems clear that the amiloride sensitive Na absorption pathway could also contribute to excessive secretion if it were down regulated or blocked (Fig 12). How can this excessive capability for fluid secretion be mitigated or avoided? Clearly we must use pharmacological or gene therapy techniques to decrease secretion via the cAMP - dependent pathway or up-regulate or increase fluid absorption via the apical membrane Na - dependent pathways. Over the next year we will carry out a variety of studies on these mammary cells that are designed to remove fluid form the lumenal side of these epithelia. Important clues on how to do this have come from our previous work from the eye and lung (Jiang et al., 1997; Quong et al., 1997).

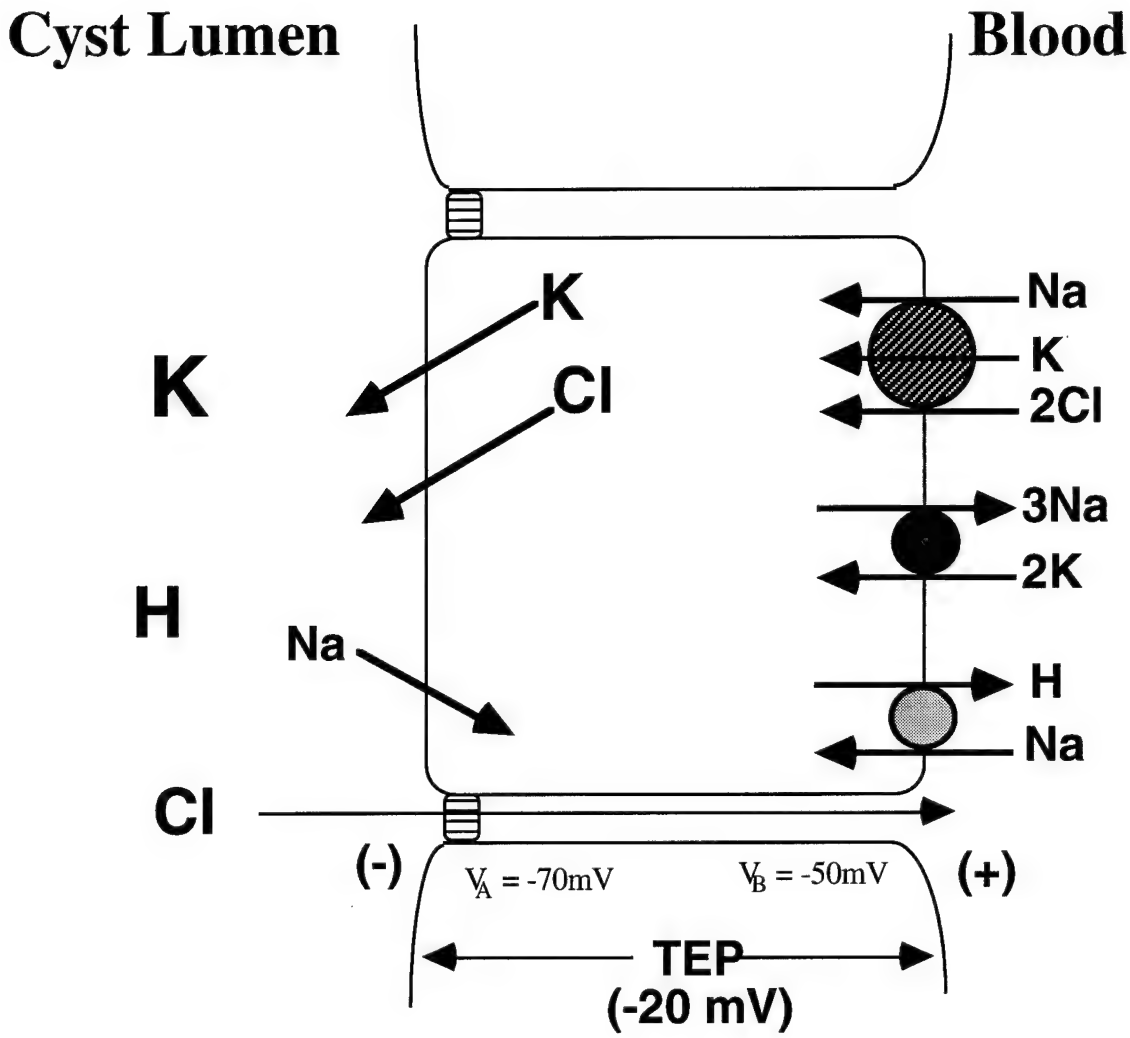

Figure 1. Ion transport model for Mammary cell.

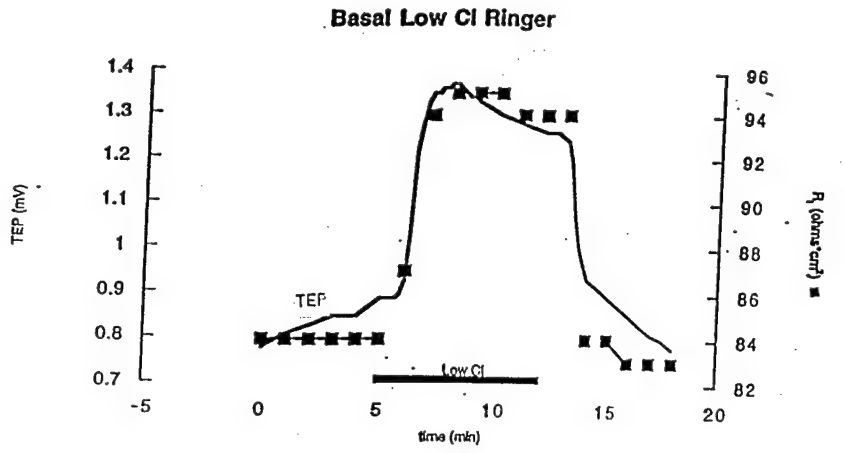

Figure 2. TEP and R_T responses of MCF-7 cells to low Cl ringer in the **basal** bath. LowCl ringer produces a rapid and reversible increase in TEP and R_T.

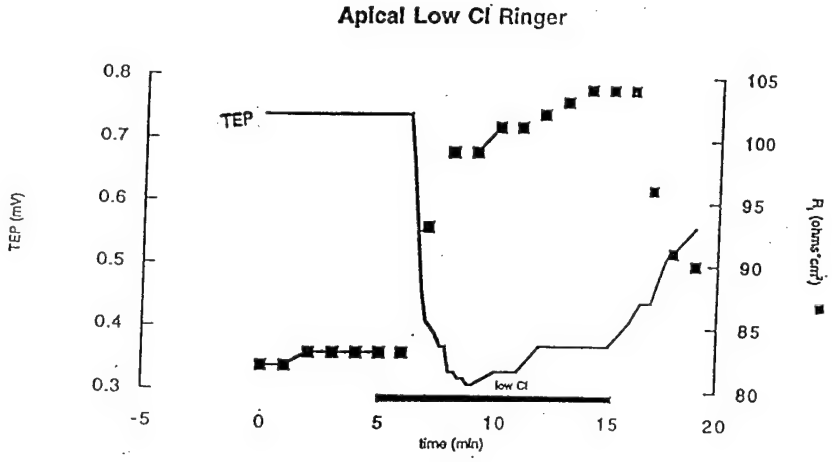

Figure 3. TEP and R_T responses of MCF-7 cells to low Cl ringer in the **apical** bath. In low Cl Ringer TEP decreased and R_T increased. These responses are reversible.

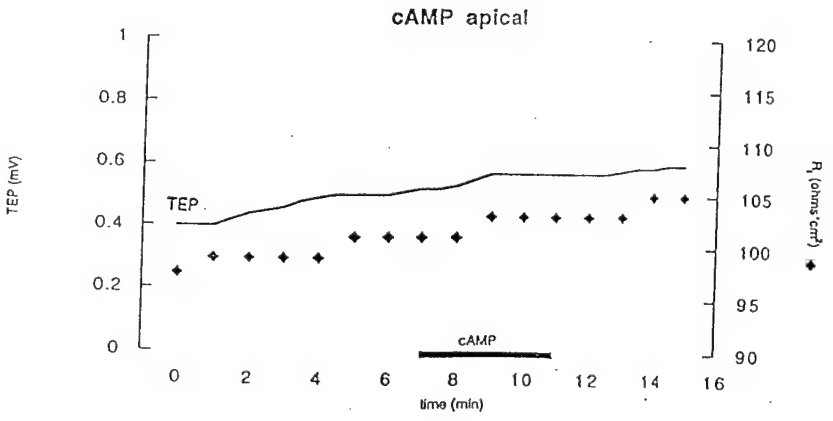

Figure 4. No response when cAMP is added to apical bath of MCF-7 cells.

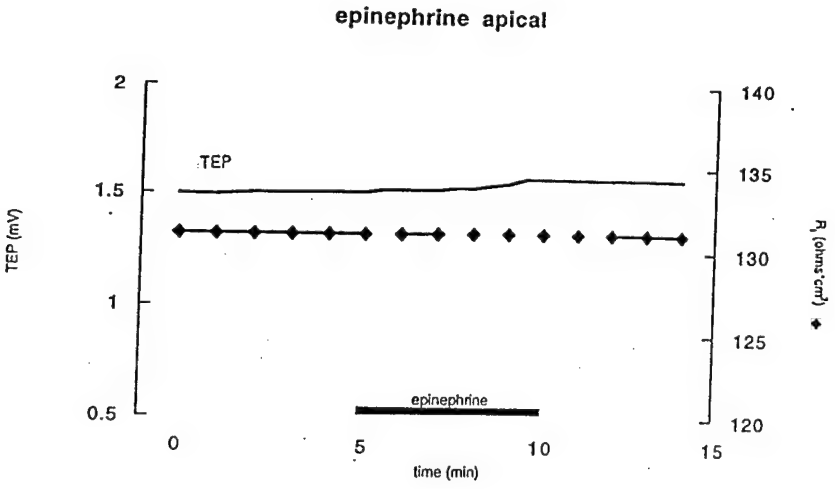

Figure 5. No response was observed with addition of epinephrine to the apical bath of MCF-7 cells.

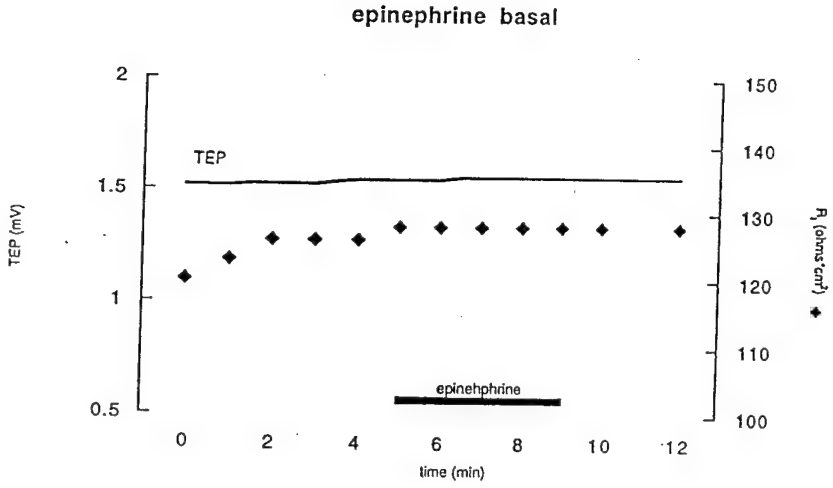

Figure 6. No response was observed with the addition of epinephrine to the basolateral bath of MCF 7-cells.

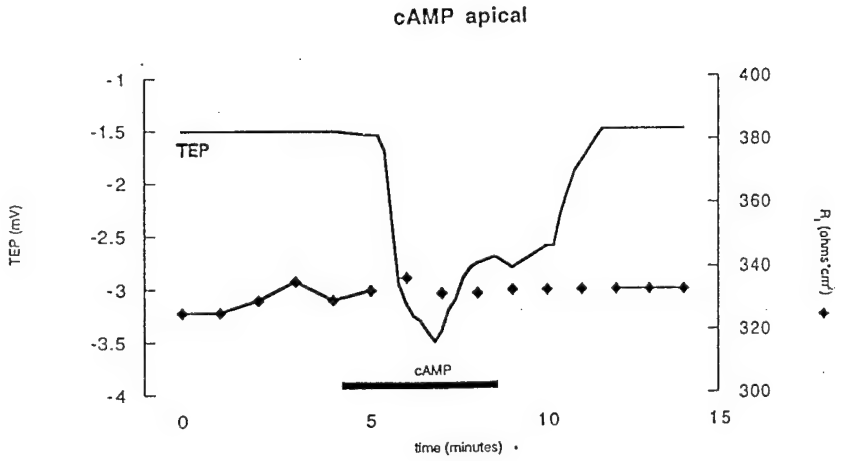

Figure 7. Addition of a cAMP cocktail to the apical bathing solution of 31EG4 cells caused a rapid increase in TEP toward more negative potentials. This increase was followed by a slower decrease toward zero; there was little change in total tissue resistance.

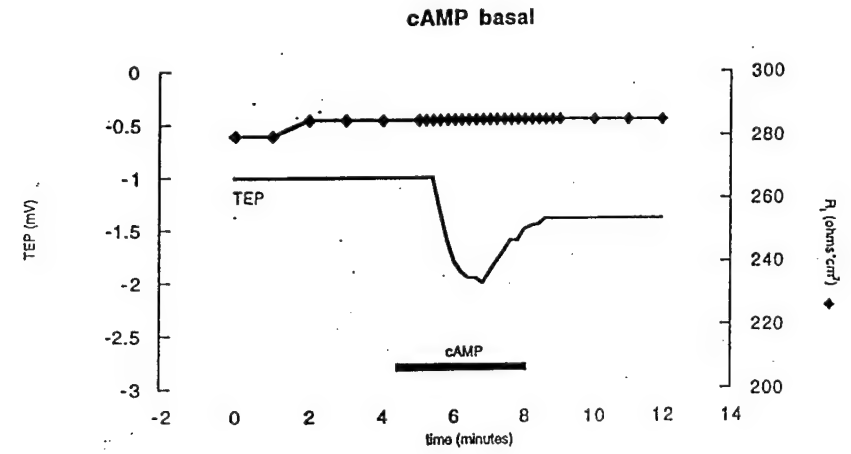

Figure 8. Addition of cAMP cocktail to the basolateral bath of 31EG4 cells increased TEP with little change in R_T.

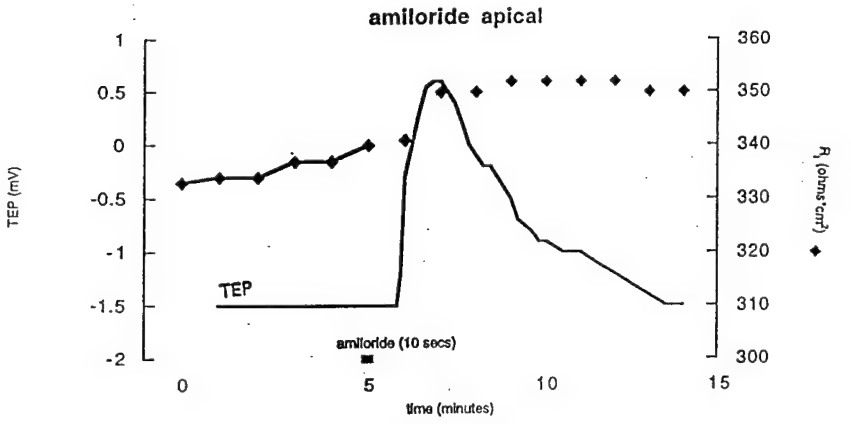

Figure 9. Addition of amiloride to the apical bath of 31EG4 cells depolarized the TEP and increased R_T consistent with the presence of amiloride sensitive sodium channels at the apical membrane. (see Fig 1.)

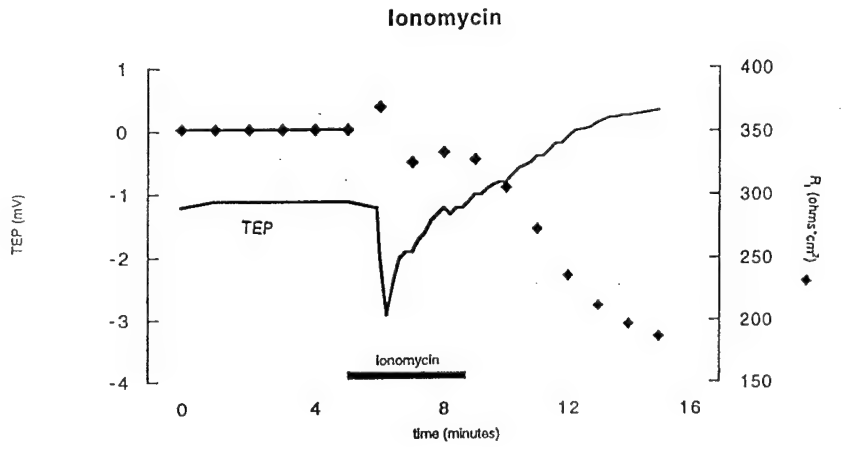

Figure 10. Addition of ionomycin, a calcium ionophore which elevates cell Ca^{2+} significantly, hyperpolarized TEP and decreased R_T. This is consistent with the presence of apical membrane Ca^{2+} sensitive Cl channels (fig. 1).

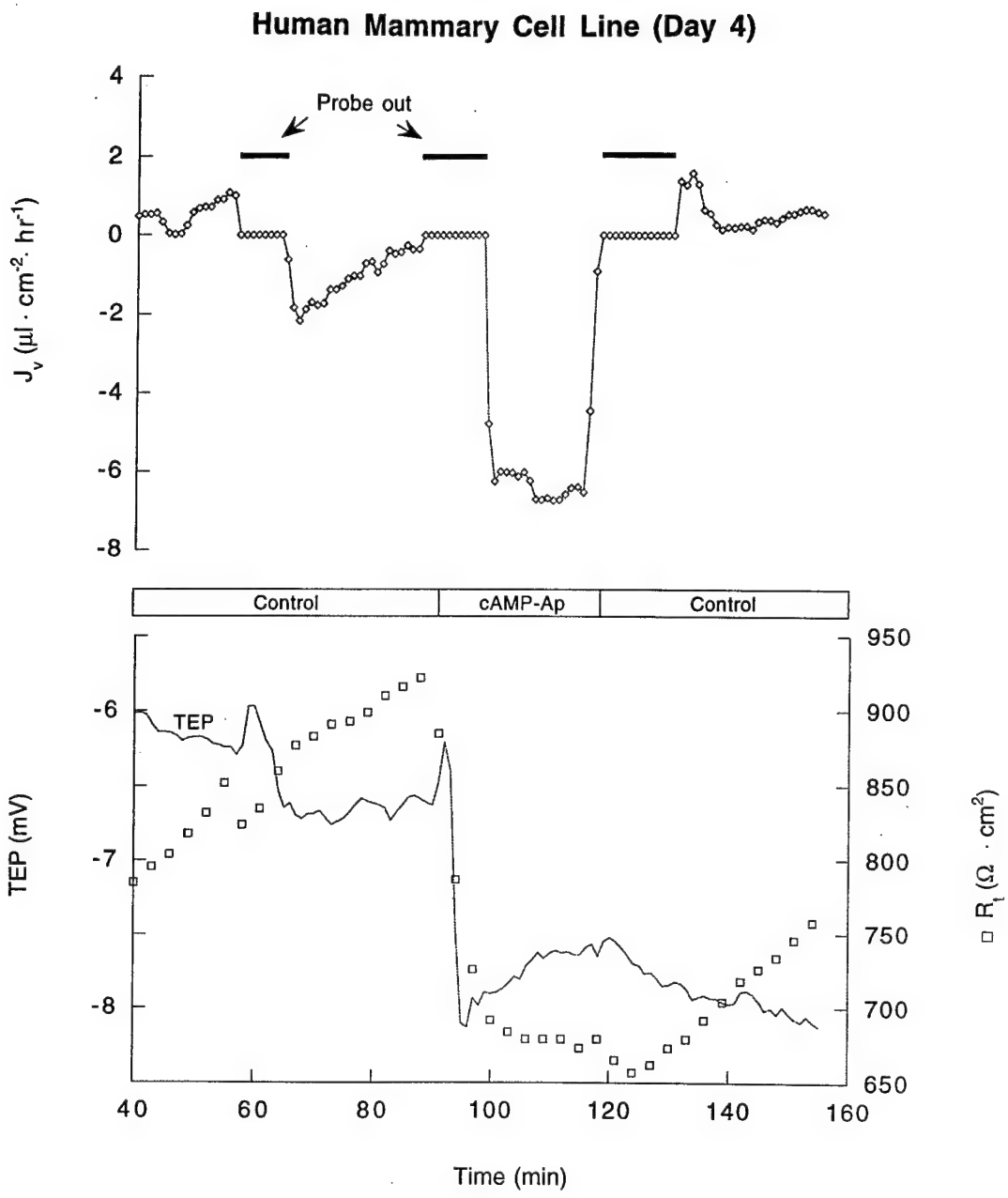

Figure 11. Three hr experiment in which voltage, resistance and fluid transport changes were measured following the addition and removal of cAMP to the apical bath of 31EG4 cells. The TEP and resistance in control Ringer; 6-7 mV and 800-900 $\Omega \cdot cm^2$, respectively. These values indicate a significant transport potential and fluid transport rate. The fluid transport rate in control Ringer was $\approx$ 2 $\mu l \cdot cm^2 \cdot hr^{-1}$ and then slowly decayed toward zero. Elevating cell cAMP caused an increase in steady-state fluid secretion to $\approx$ - 7 $\mu l \cdot cm^2 \cdot hr^{-1}$ and that increase along with the increase in TEP and the decrease in R_T suggests that the apical membranes of these cells contain cAMP-dependent Cl channels.

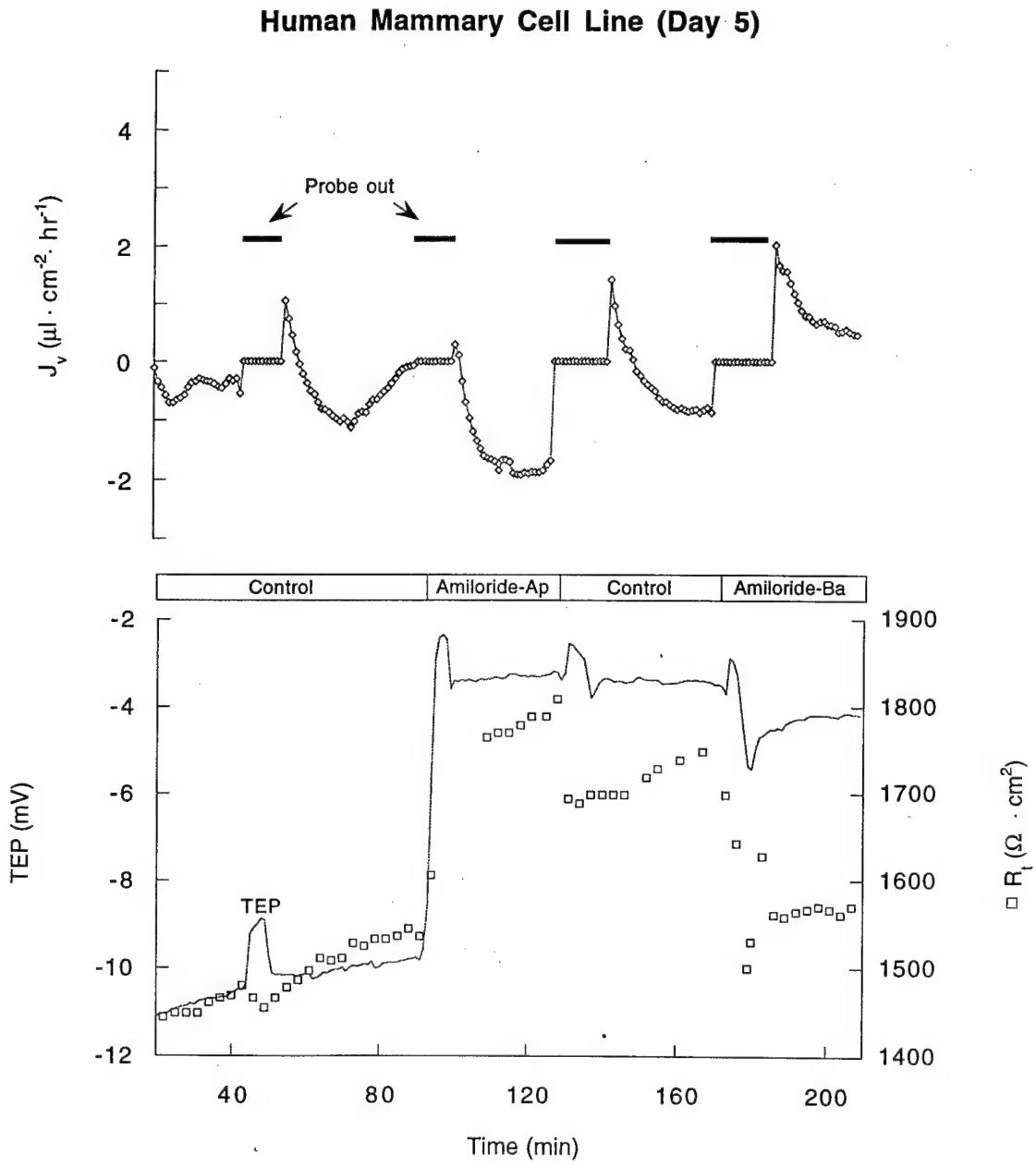

Figure 12. Three hr experiment in which voltage, resistance and fluid transport changes were measured following the addition and removal of amiloride to the apical bath of 31EG4 cells. In control Ringer the TEP and R_T are, -10 mV and 1500 $\Omega \cdot cm^2$. The fluid transport rate in control Ringer was initially -1 $\mu l \cdot cm^2 \cdot hr^{-1}$ and then decayed toward zero (note the following control held steady at (-1 $\mu l \cdot cm^2 \cdot hr^{-1}$). The addition of amiloride to the apical bath caused a rapid and large decrease of TEP (-10 to -3 mV) and a large increase in R_T ($\approx$ 300 $\Omega \cdot cm^2$). At the same time J_V increased to $\approx$ -2 $\mu l \cdot cm^2 \cdot hr^{-1}$.

Control				
Filename	Jv backg.	Abs/Sec	TEP	Rt
09177			-1.97	320
09237	-1.4	sec	-0.45	215
09247	-2.25	sec	-0.35	180
09297hma	0	NA	-5.4	880
09297hmb	23	abs	-9.5	1150
09297hmc	10	abs	-8	1100
09307	-0.5	sec	-10.5	1500
10017	17	abs	-8.5	1250
10087	1.7	abs	-12	750
10107	-1.167	sec	-10	740
10147	-0.7	sec	-4.1	470

Statistics		
	average	st dev
Jv - all	4.5683	8.952472
TEP-all	-6.43364	4.190674
Rt - all	777.7273	444.7435
Jv - sec	-1.2034	0.686209
TEP - sec	-5.08	4.958654
Rt - sec	621	12.81653
Jv - abs	12.925	9.177282
TEP - abs	-9.5	1.779513
Rt - abs	1062.5	217.4665

T-test P-value abs. Vs. sec	
Jv	0.053836
TEP	0.12121
Rt	0.151516

cAMP				normalized	
Filename	Delta Jv	Delta TEP	Delta Rt	%mx TEP	%mx Rt
09177	N/A	-0.7	-50	35.53299	-15.625
09237	N/A	-0.8	-55	177.7778	-25.5814
09297a	N/A	-2	-240	37.03704	-27.2727
09297b	N/A	-1.8	-400	18.94737	-34.7826
09297c	N/A	-1.9	-270	23.75	-24.5455
10147	N/A	-0.8	-110	19.5122	-23.4043

	apical NPPB after		
	delta Jv	delta TEP	delta Rt
09177	N/A	N/A	N/A
09237	N/A	N/A	N/A
09297a	N/A	N/A	N/A
09297b	N/A	6	150
09297c	N/A	6	150
10147	N/A	2.6	?

	statistics			
	Delta TEP	Delta Rt	%mx TEP	%mx Rt
average	-1.33333	-187.5	52.0929	-25.2019
stdev	0.625033	139.4184	62.06617	6.190344

Table1. The preliminary fluid transport and physiology data from 31EG4 cells.

LITERATURE CITED

Altenberg GA, Deitmer JW, Glass DC, Reuss L. P-glcoprotein-associated Cl^- currents are activated ab cell swelling but do not contribute to cell volume regulation. *Cancer Research.* 1994; 54: 618-622.

Angeli A; et al,. Categorization of cysts and steroid levels in breast cyst fluid. *Cancer Detection and Prevention.* 1992; 16(1):53-6.

Angeli A; Dogliotti L; Naldoni C; Orlandi F; Puligheddu B; Caraci P; Bucchi L; Torta M; Bruzzi P. Steroid biochemistry and categorization of breast cyst fluid: relation to breast cancer risk. *Journal of Steroid Biochemistry and Molecular Biology*, 1994; 49(4-6):333-9.

Angeli A, Dogliotti L, Orlandi F. Beccati D. Mammary cysts: pathophysiology and biochemistry. *Nucl. Med. Biol.* 1987; 397-406.

Athanassiadou PP; Veneti SZ; Kyrkou KA; Intzes KS; Mouzaka LI; Papadimitriou GK. Presence of epidermal growth factor receptor in breast cancer smears of cyst fluids: relationship to electrolyte ratios and pH concentration. *Cancer Detection and Prevention*, 1992; 16(2): 113 - 118.

Bialek S; Miller SS. K+ and Cl- transport mechanisms in bovine pigment epithelium that could modulate subretinal space volume and composition. *Journal of Physiology*, 1994 Mar 15; 475(3):401-17.

Bodian CA. Benign breast diseases, carcinoma in situ, and breast cancer risk. *Epidemiologic Reviews*, 1993; 15(1):177-87.

Bodian CA; Lattes R; Perzin KH. The epidemiology of gross cystic disease of the breast confirmed by biopsy or by aspiration of cyst fluid. *Cancer Detection and Prevention*, 1992; 16(1):7-15.

Bundred NJ; West RR; Dowd JO; Mansel RE; Hughes LE. Is there an increased risk of breast cancer in women who have had a breast cyst aspirated? *British Journal of Cancer*, 1991 Nov; 64(5):953-5.

Bradlow HL, fleisher M, Schwartz MK, Nisselbaum J, Breed CN. classification of patients withgross cystic breast disease according to the biochemical composition of breast cyst fluid. In: *Fibrocystic Breast Disease.* Ediitio Cantor, Aulendorf; 1985; 9-20.

Cassoni P; Sapino A; Haagensen DE; Naldoni C; Bussolati G; Mitogenic effect of the 15 - kDa gross cystic disease fluid protein (GCDFP - 15) on breast - cancer cell lines and on immortal mammary cells. *International Journal of Cancer*, 1995; 60(2): 216 - 220.

Ciatto S; et al,. Risk of breast cancer subsequent to proven gross cystic disease. *European Journal of Cancer*, 1990; 26(5):555-7.

Dogliotti L, Orlando F, Caraci P, Puligheddu B, Torta M, Angeli A. Biochemistry of breast cyst fluid: an approach to understanding intracellular communication in the terminal duct lobulat units. Ann. N. Y. Acad. Sci. 1990; 586: 17-28.

Edelman JL; Miller SS. Epinephrine stimulates fluid absorption across bovine retinal pigment epithelium.*Investigative Ophthalmology and Visual Science*, 1991 Nov; 32 (12) : 3033 -40.

Edelman JL; Lin H; Miller SS. Acidification stimulates chloride and fluid transport across frog retinal pigment epithelium. 1994a; *American Journal of Physiology.* 1994; 266 (*Cell Physiol.* 35): C946-956.

Edelman JL; Lin H; Miller SS. Potassium-induced chloride secretion across the frog retinal pigment epithelium. *American Journal of Physiology*, 1994b; 266 (*Cell Physiol.* 35): C957-966.

Eidelman S, Damsky CH, Wheelock MJ, Damjanov, I. Expression of the cell-cell adhesion glycoprotein cell-CAM 120/80 in normal human tissue and tumors. American J. Pathology, 1989 35: 101-110.

Flezar M, Heisler S. P2-purinergic receptors in human breast tumor cells: coupling of intracellular calcium signaling to anion secretion. *Journal of Pharmacology and Experimenntal Therapeutics.* 1993; 265 (3): 1499-1510.

Furuya K, Enomoto K, Yamagishi S. Spontaneous calcium oscillations and mechanically and chemically induced calcium responses in mammary epithelial cells. *Pflugers Archiv European Journal of Physiology.* 1993; 422(4): 295-304.

Gallemore, R.P., Hughes, B.A. and Miller S.S. Retinal pigment epithelial transport mehanisms and their contributions to the electroretinogram. In: *Progress in retinal and Eye Research* , 1997, 16: 509 - 566.

Hughes BA; Miller SS; Machen TE. Effects of cyclic AMP on fluid absorption and ion transport across frog retinal pigment epithelium. Measurements in the open-circuit state. *Journal of General Physiology*, 1984 Jun; 83(6):875-99.

Imagawa W, Yang J, Guzman r, Nandi S. Control of Mammary Gland Development. In: Knobil E, Neil JD, eds *The Physiology of Reproduction,* Second Edition New York, raven Press. 1994; 1033-1063.

Jiang C; Finkbeiner WE; Widdicombe JH; and Miller SS. Fluid transport across cultures of himan tracheal glands is altered in cystic fibrosis. Journal of Physiology 1997 15; 501:637-647.

Jiang C; Finkbeiner WE; Widdicombe JH; McCray PB Jr; Miller SS. Altered fluid transport across airway epithelium in cystic fibrosis. Science, 1993 Oct 15; 262(5132):424-7.

Lai LC; et al,. Growth inhibitory substances in human breast cyst fluid. *Anticancer Research*, 1993 Jan-Feb; 13(1):225-7.

Lai LC; Kadory S; Cornell C; Lennard TW. Possible regulation of soluble ICAM-1 levels by interleukin-1 in a sub-set of breast cysts. *International Journal of Cancer*, 1993 Oct 21; 55(4):586-9.

Leis HP. Gross Breast Cysts. Significance of apocrine type, identification by cyst fluid analysis and management. *Breast Disease*, 1993; 6:185-194.

Lin H; Miller SS. pHi-dependent Cl-HCO3 exchange at the basolateral membrane of frog retinal pigment epithelium. *American Journal of Physiology*, 1994; 266:C935-45.

Marcus DC; Takeuchi S; Wangemann P. Ca(2+)-activated nonselective cation channel in apical membrane of vestibular dark cells. *American Journal of Physiology*, 1992 Jun; 262(6 Pt 1):C1423-9.

Mazolijian G, Bodian C, Haagensen DE, Haagensen CD. Expression of GCDFP-15 in breast carcinomas. *Cancer.* 1989; 63: 2156-2161.

Miller WR. The biochemistry of cyst fluids. *Br. J. Clin. Pract.* 1989; Suppl. 68: 94-99

Molina R, Filella X, Herranz M, et al, Biochemistry of cyst fluid in fibrocystic disease of the breast. Approach to classification and understanding of the mechanism of formation. *Annals of the New York Academy of Sciences*, 1990; 586:29-42.

Peterson, W.M., Meggyesy C., Yu, K. and Miller S.S. Extracellular ATP activates calcium signalling, ion and fluid transport in retinal pigment epithelium. The Journal of Neuroscience, 1997, 17(7): 2324 - 2337.

Petrakis NL; Lowenstein JM; Wiencke JK; Lee MM; Wrensch MR; King EB; Hilton JF; Miike R. Gross cystic disease fluid protein in nipple aspirates of breast fluid of Asian and non-Asian women. *Cancer Epidemiology, Biomarkers and Prevention*, 1993 Nov; 2(6):573-9.

Quong J, Miller SS., Genetic Alteration of Retinal Pigment Epithelium Function. *ARVO*, 1997 March. Vol. 38(4):s258.

Reed MJ, Coldham NG, Patel SR, Ghilchic MW, James VHT. Interleukin-1 and Interleukin-6 in breast cyst fluid: their role in regulating aromatase activity in breast cancer cells. *J. Endocr.* 1992; R5-R8.

Richards J, et al.. Method for culturing mammary epithelial cells in a rat tail collagen gel matrix. *Journal of Tissue Culture Methods.* 1984; Vol. 8(1): 31-36.

Shennan DB. Evidence for furosemide-sensitive Na^{+}-K^{+}-Cl^{-} co-transport in lactating rat mammary tissue. *Q.J. Exp. Physiol.* 1989; 74: 927-938.

Shennan DB. Mechanisms of mammary gland ion transport. *Comparative Biochemistry and Physiology. a: Comparative Physiology.* 1990; 97(3):317-24.

Shennan DB. K and Cl transport by mammary secretiry cell membrane vesicles isolated from milk. *Journal of Dairy Research.* 1992; 59(3): 339-348.

Shiozaki H, Tahara H, Oka H, et al., Expression of immunoreactive E-cadherin adhesion molecules in human cancers. American J. Pathology 139: 17-23.

Sjaasted MD, Zettl KS, Parry G, Firestone GL, Machen TE. hormonal regulation of the polarized function and distribution of Na/H exchange and Na_HCO_3 cotransport in cultured mammary epithelial cells. *Journal of Cell Biology.* 1993; 122: 589-600.

Umaar R; et al,. Does the protein composition of breast secreations alter in malignant breast disease? 17th Annual San Antonio Breast Cancer Symposium. December 1994.